



US005824550A

United States Patent [19]**Hruska et al.**[11] **Patent Number:** **5,824,550**[45] **Date of Patent:** **Oct. 20, 1998**

[54] **VOLTAGE-GATED CALCIUM CHANNEL AND ANTISENSE OLIGONUCLEOTIDES THEREO**

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[21] Appl. No.: **330,433**

[22] Filed: **Oct. 28, 1994**

[51] **Int. Cl.**⁶ **C12N 5/10**; C07H 21/00; C12Q 1/68

[52] **U.S. Cl.** **435/375**; 435/6; 536/23.5; 536/24.3; 536/24.31; 536/24.33; 536/24.5

[58] **Field of Search** 536/23.5, 24.3, 536/24.31, 24.33, 24.5; 514/44; 435/6, 240.2, 375; 935/33, 34, 36, 8, 9, 11

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[57] **ABSTRACT**

An antisense oligonucleotide of 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the SA-Cat channel gene DNA or mRNA is provided, together with pharmaceutical compositions containing and methods utilizing such antisense oligonucleotide.

11 Claims, 6 Drawing Sheets

FIG. 1

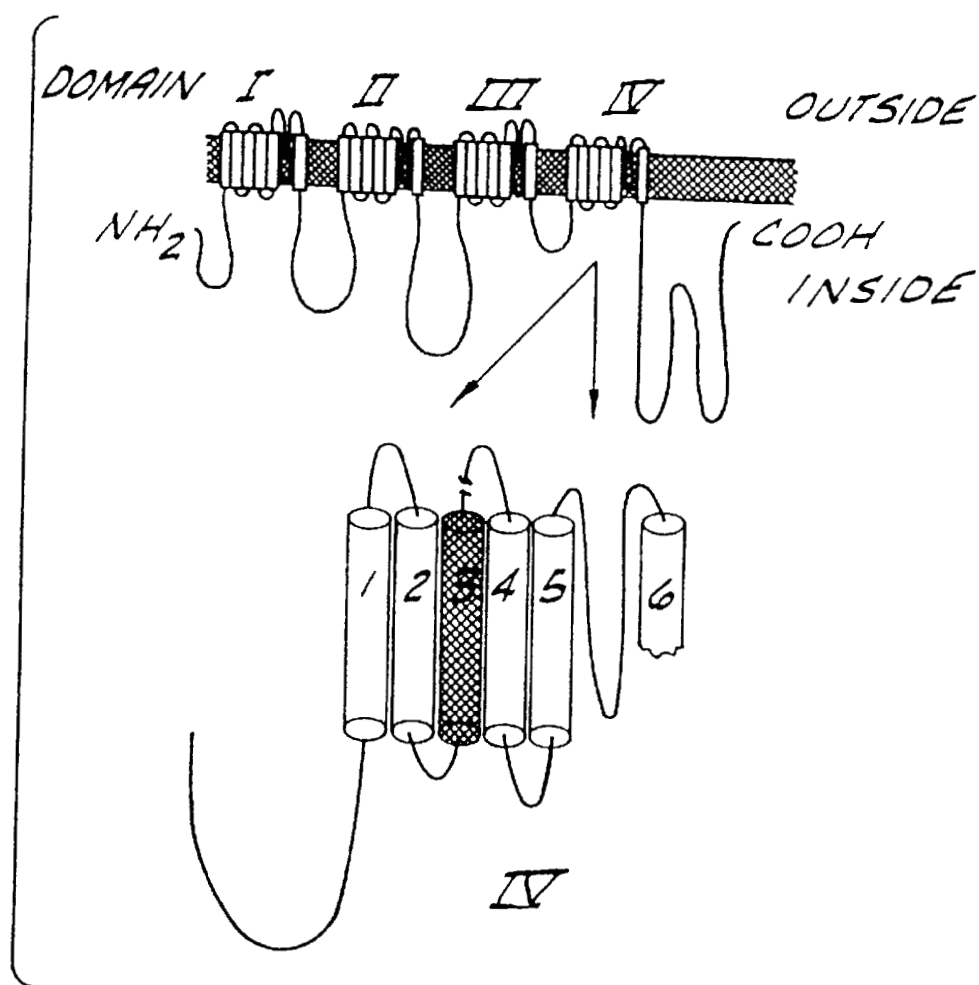


FIG. 2

ROB1 (SEQ ID NO: 7)	PWNVFDLIVIGSIIDVILSEID.....DPDESARISSAFFRLFRVMRLIKLLSRA
RabSkel1	PWNVFDLIVIGSIIDVILSEIDTFLASSGGLYCLGGCGNVPDESARISSAFFRLFRVMRLIKLLSRA
(SEQ ID NO: 8)	
ROB2 (SEQ ID NO: 9)	PWNVFDLIVIGSIIDVILSETN.....SAEENSRIISITFFRLFRVMRLVKLLSRG
RatBr2 (SEQ ID NO: 10)	PWNVFDLIVIGSIIDVILSETNPAEHTQCPSM.....SAEENSRIISITFFRLFRVMRLVKLLSRG
ROB3 (SEQ ID NO: 11)	AWNTFDSLIVIGSIIDVALSEAD.....NSEENRISITFFRLFRVMRLVKLLSRG
RatBr3 (SEQ ID NO: 12)	AWNTFDSLIVIGSIIDVALSEADPSDSENIPLTATPG.....NSEENRISITFFRLFRVMRLVKLLSRG
	IVS3
	IVS4

FIG. 3

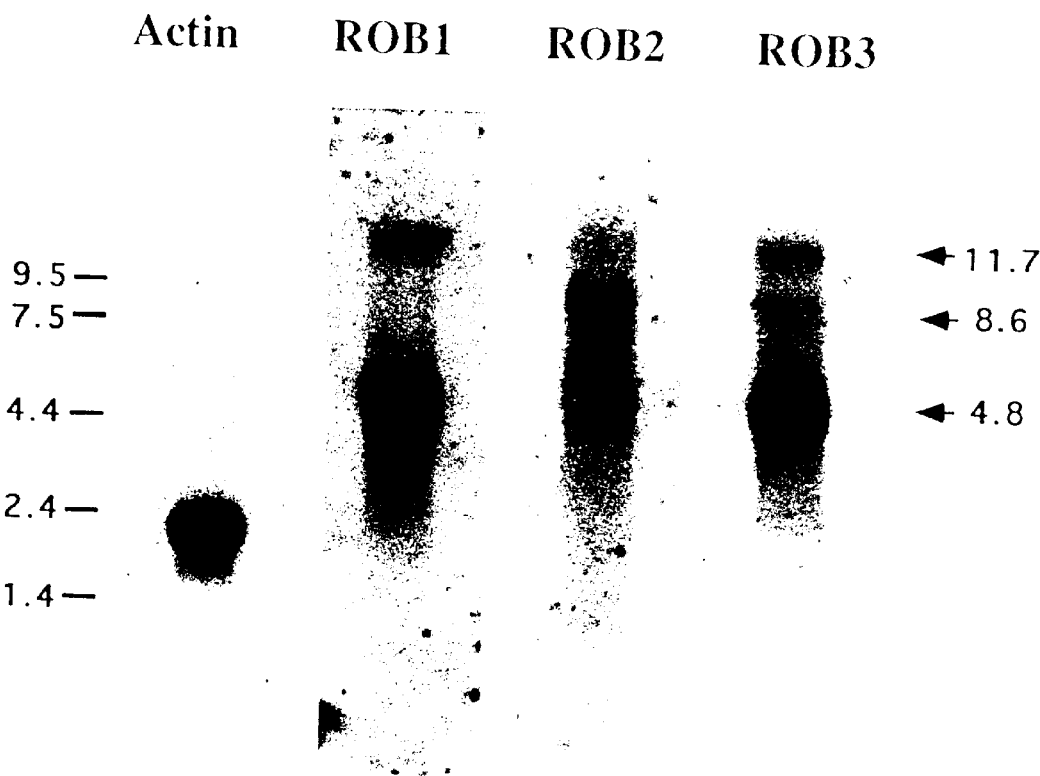


FIG. 4a

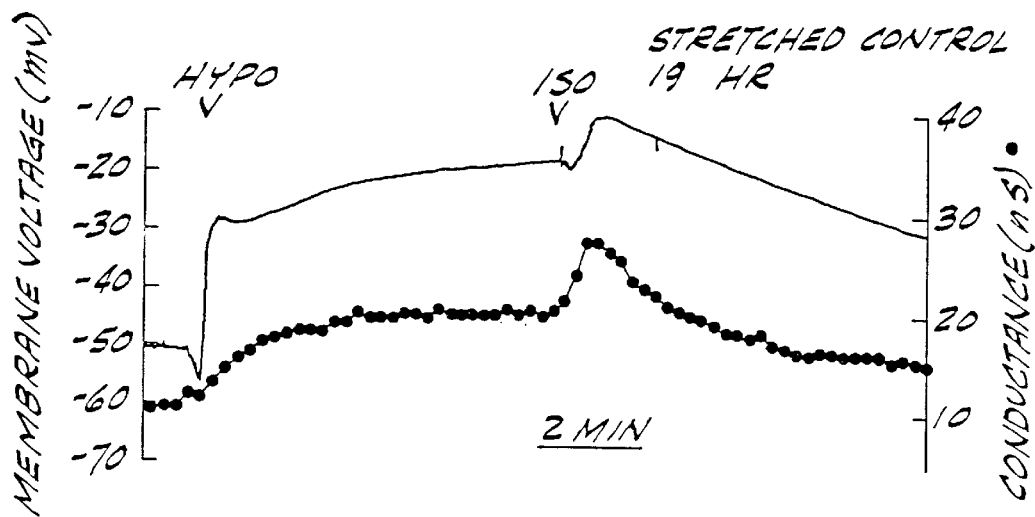


FIG. 4b

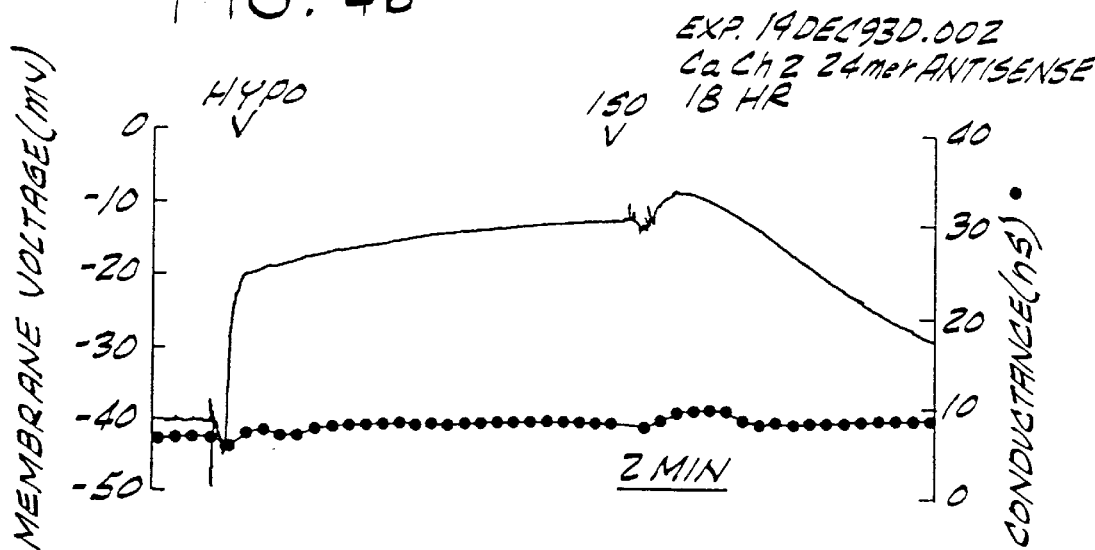


FIG. 5A

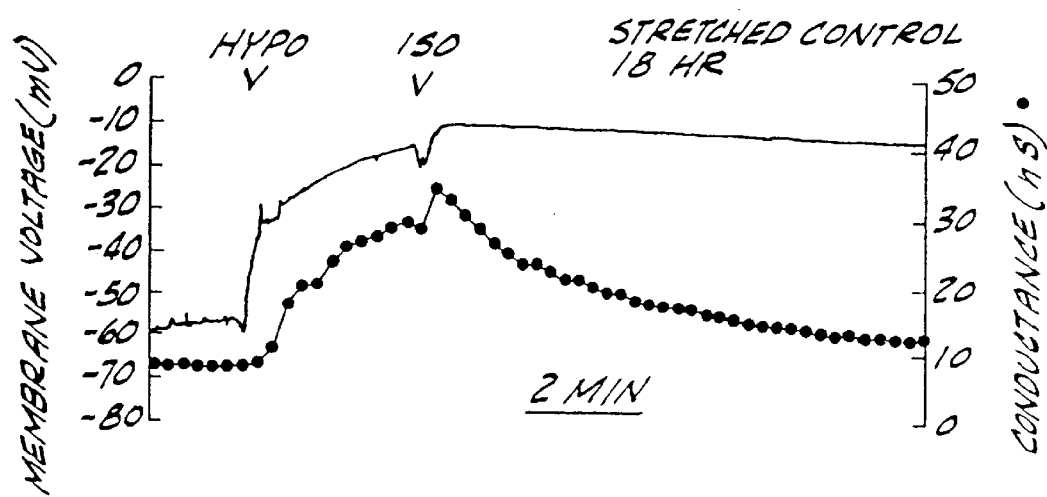


FIG. 5B

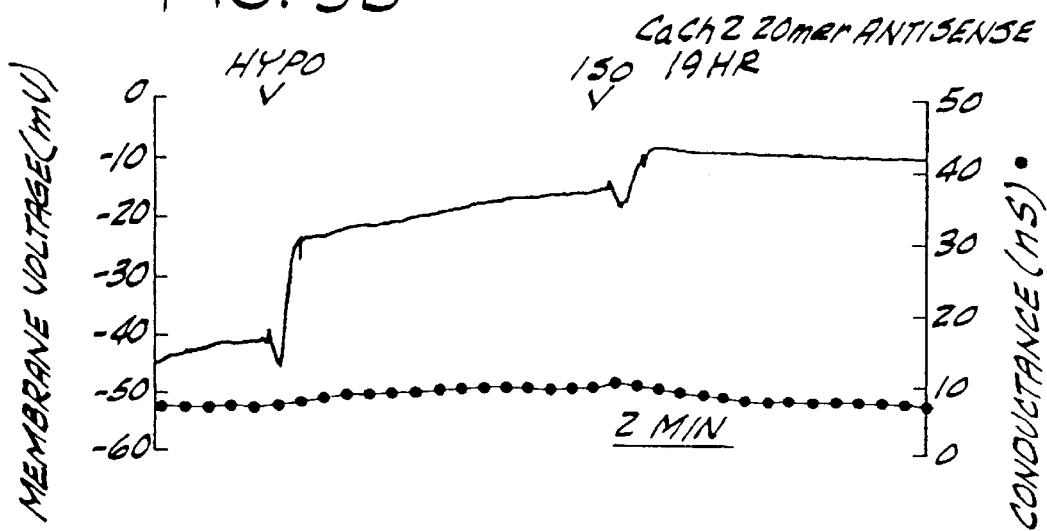
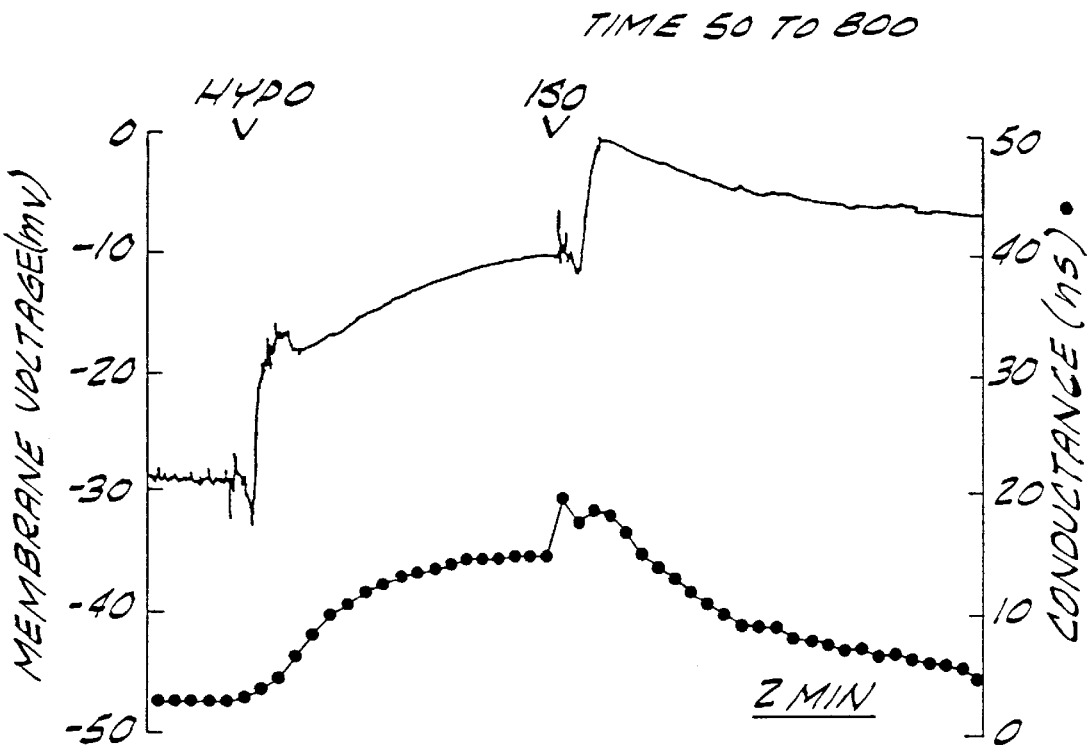


FIG. 6



VOLTAGE-GATED CALCIUM CHANNEL AND ANTISENSE OLIGONUCLEOTIDES THERE TO

This invention was made with Government support under NASA grant NAG 2-791 awarded by the National Aeronautics and Space Administration and NIH grants AR 39561 and GM 34399 awarded by the National Institute of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

This invention relates to a voltage-gated calcium channel that stimulates bone formation in osteoblasts and mediates arterial tone in endothelial cells. More specifically, it relates to the gene for the voltage-gated calcium channel, antisense oligonucleotides useful for regulating the expression of the voltage-gated calcium channel gene and processes for the use of the antisense oligonucleotides to inhibit expression of the voltage-gated calcium channel, to inhibit bone formation, to mediate vascular blood pressure, and as an analytical tool useful for the development of strategies for responding to the bone loss caused by osteoporosis.

It has been known for a century that mechanical strain—whether resulting from physiological exercise or artificial means—increases bone formation and remodeling activity resulting in increased bone mass. Similarly, endothelial cells subjected to mechanical forces via fluid shear and vessel expansion have been shown to undergo increased cell proliferation and vasoactive secretions. Prior to the present invention, however, the precise molecular mechanism for enhancing the amount of bone deposited at the vector of force applied in osteoblasts and for increasing endothelial cell proliferation and vasoactive secretion, has remained unclear.

Voltage-gated calcium channels are members of a superfamily of cation channels, which also includes channels for Na^+ , K^+ and divalent cations, i.e., Ca^{2+} . Calcium channels are responsible for the rapid entry of calcium into excitable cells upon membrane depolarization and are essential for excitation-contraction coupling in muscle, and the propagation of action potentials and neurotransmitter release in neurons. In addition, calcium channels regulate stimulus-secretion coupling during hormone release in endocrine glands. Much less is known about the roles of these calcium channels in other types of nonexcitable tissues.

Calcium channels may be sub-classified according to their electrophysiological behavior and pharmacological sensitivities. L-type calcium channels are defined pharmacologically by their sensitivity to three classes of calcium channel blockers, the phenylalkylamines, benzothiazepines and dihydropyridines. L-type calcium channels are multi-component proteins made up of five subunits: α_1 , α_2 , δ , γ , and β . The properties of calcium channels are largely conferred by the α_1 subunit, which forms the actual pore site for the channel. The structure of the α_1 subunit has been elucidated. It consists of four homologous repeats (I–IV) consisting of six membrane-spanning domains (S1–S6). The dihydropyridine receptor for the L-type channel is located on the carboxyl end of the α_1 unit of the protein just beyond the S6 domain of the IV region.

Although common features of the structure of calcium channels have been established, variations in this structure have also been identified. At least six classes of calcium channel α_1 subunit genes have now been identified, three of which are members of the dihydropyridine-sensitive L-type subfamily, including skeletal muscle (CaCh1), cardiac

muscle (CaCh2) and neuroendocrine (CaCh3) isoforms. Still greater diversity is generated by alternative splicing.

Osteoblasts, the cells responsible for bone growth, have voltage-gated calcium (SA-Cat) channels that are known to play a role in bone formation. Recent studies indicate that a similar voltage-gated calcium channel is present in endothelial cells. Stretch-activated cation channels are, as the name suggests, activated by mechanical stimuli, e.g., mechanical loading or osmotic change. SA-Cat channels are voltage-independent, gadolinium-inhibitable and cation non-selective. In the present patent application we identify the osteoblast voltage-gated calcium channel as a member of the calcium channel superfamily, namely a CaCh2 isoform.

Osteoporosis is a debilitating disease characterized by the loss of normal bone density with the thinning of bone tissue and the growth of small holes in the bones. Osteoporosis frequently causes chronic pain, especially in the lower back, increased frequency of broken bones, loss of body height, and eventual loss of mobility and other body function. Osteoporosis has been classified into two categories. Primary osteoporosis, which includes post-menopausal osteoporosis, and secondary osteoporosis, which includes immobilization or disuse osteoporosis, together afflict approximately 20 million Americans.

Osteosclerosis is a lesser-known condition characterized by an abnormal increase in the density of bone mass. The condition occurs in various diseases and is often linked to poor circulation in the bone tissue, infection or tumor formation.

Hypertension is a common, generally asymptomatic disease marked by high blood pressure persistently exceeding 140/90. Essential hypertension, also known as primary hypertension, has no single known cause, and is the most prevalent form of hypertension, having been diagnosed according to some estimates, in over 50 million Americans. Secondary hypertension is linked to disease of the kidneys, lungs, glands or vessels. For example, pulmonary hypertension is a condition of unusually high pressure within the blood vessels of the lungs.

Antisense oligonucleotides are short synthetic nucleotide sequences formulated to be complementary to a portion of a specific gene or mRNA. They function by hybridizing to complementary sequences, resulting in selective arrest of expression of the complementary gene or mRNA. In particular, the cytoplasmic location of mRNA provides a readily accessible target for antisense oligonucleotides entering the cell. In addition to the use of antisense oligonucleotides as therapeutic agents due to their ability to block expression of a specific target protein, they also provide a useful tool for exploring regulation of the expression of a gene of interest in vitro and in tissue culture (see Rothenberg, M., et al., *Natl. Cancer Inst.*, 81:1539–1544, 1989).

The development of new therapeutic strategies against, and the creation of new analytical tools for, a better understanding of diseases such as osteoporosis, osteosclerosis, and hypertension, are greatly desired. It is particularly desirable to provide such tools and therapies that are highly specific to a target gene and protein, which at the same time, because of their specificity, do not substantially affect other proteins or body functions. The provision of antisense oligonucleotides for use in designing therapies and diagnostic tools for diseases and physiological responses related to voltage-gated calcium channel mediated physiological activities satisfies a long-sought need for such therapies and tools.

SUMMARY OF THE INVENTION

Among the objects of the invention, therefore, may be noted the provision of antisense oligonucleotides and pharmaceutical compositions that are capable of inhibiting the function of the voltage-gated calcium gene or mRNA and the provision of such oligonucleotides and compositions that serve as useful tools for exploring the regulation of gene expression in vitro and in tissue culture for the voltage-gated calcium. Also provided are a method for selectively blocking transcription or translation of the voltage-gated calcium channel gene and mRNA, for halting disease processes associated with the expression of that gene, and therapeutic procedures for the treatment of osteosclerosis and hypertension.

Briefly, therefore, the present invention is directed to an antisense oligonucleotide of 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA.

In a further embodiment, the invention is directed to a pharmaceutical composition comprising at least one antisense oligonucleotide of 10 to 35 nucleotides in length that is capable of hybridizing with a region of the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA and a pharmaceutically acceptable carrier.

In another aspect of the present invention, a method of inhibiting expression of the voltage-gated calcium channel gene is provided. The method comprises the steps of obtaining an antisense oligonucleotide of 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA, combining the antisense oligonucleotide with a pharmaceutically acceptable carrier to create a pharmaceutically active mixture, and contacting the pharmaceutically active mixture with the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA, thereby inhibiting expression of the voltage-gated calcium channel protein.

In yet another embodiment of the instant invention, a method of treating hypertension using the antisense oligonucleotides to the voltage-gated calcium channel gene is provided. The method includes the steps of obtaining a hypertensive-reductive amount of an antisense oligonucleotide 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the SA-Cat channel gene DNA or mRNA, combining the hypertensive-reductive amount of the antisense oligonucleotide with a pharmaceutically acceptable carrier to create a pharmaceutically active mixture, and contacting the pharmaceutically active mixture with the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA of endothelial cells lining the blood vessels, inhibiting expression of the voltage-gated calcium channel protein and thereby causing a reduction in blood pressure.

In a further embodiment of the instant invention, a method of treating osteosclerosis using the antisense oligonucleotides to the voltage-gated calcium channel gene is provided. The method includes the steps of obtaining an osteosclerotic-reductive amount of an antisense oligonucleotide 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA, combining the osteosclerotic-reductive amount of the antisense oligonucleotide with a pharmaceutically acceptable carrier to create a pharmaceutically active mixture, and contacting the pharmaceutically active mixture with the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA of osteoblasts in a target tissue, inhibiting expression of the voltage-gated

calcium channel protein and thereby causing a reduction in bone density in the target tissue.

Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

SEQ ID NO.1 depicts the cDNA encoding for a region of the α_1 subunit of the voltage-gated calcium channel protein.

FIG. 1 is a schematic depiction of a model of an L-type calcium channel α_1 subunit such as the voltage-gated calcium channel of the invention. The channel is composed of four homologous domains (I-IV) that are modeled to contain six transmembrane regions (S1-S6), and a segment (H5 or SS1-SS2) between the S5 and S6 domains, which is thought to loop into the membrane and line the external entrance to the pore of the channel. The IVS3 region is shaded to indicate that alternative splicing can generate two variants of this region. The hatched line just downstream of the S3 region indicates the site where a segment is omitted in the bone transcripts as a result of alternative splicing.

FIG. 2 depicts a comparison of the deduced amino acid sequences of the UMR-106 clone with its corresponding CaCh isoform in the region where alternative splicing generates a deletion (ROB2 to the rat brain CaCh2 transcript (RatBr2)).

FIG. 3 is an autoradiogram of a Northern blot of RNA hybridized to UMR-106, calcium channel riboprobes. A riboprobe corresponding to clones ROB2 was hybridized to poly (A^+) RNA (10 μ g/lane) isolated from UMR-106 cells. The actin riboprobe was hybridized to one-tenth as much RNA (1 μ g/lane). Autoradiography was for 67 hours (ROBs) or 6 hours (actin). The actin riboprobe, which was used as a control, hybridized to a single mRNA band of approximately 2.0 kB. An RNA ladder (Gibco/BRL) was used for size markers (in Kilobases). FIGS. 4(a) and 4(b) are graphic representations of the effect of a preferred antisense 24mer oligonucleotide from the α_1 subunit of the voltage-gated calcium channel gene (SEQ ID NO.:2) on the response of membrane potential and whole cell conductance to hypotonic stretch. FIG. 4(a) depicts the UMR-106 cell membrane potential and whole cell conductance response to hypotonic stretch and reversal to isotonic media. The cells had been exposed to chronic cyclic strain for nineteen hours. FIG. 4(b), depicts the same information as in FIG. 6(a), except that the cells had been loaded with the antisense oligonucleotide (SEQ ID NO.:2).

FIGS. 5(A) and 5(B) are graphic representations of the effect of a preferred antisense 20mer oligonucleotide from the α_1 subunit of the voltage-gated calcium channel gene (SEQ ID NO.:3) on the response to hypotonic stretch. FIG. 7(a) depicts the UMR-106 cells exposed to anti-streptolysin O but without exposure to the antisense oligonucleotide, exposed to 18 hrs of chronic cyclic strain prior to hypotonic stretch. FIG. 5(B) depicts the same procedure, but with exposure of the cells to the 20mer antisense oligonucleotide during the permeabilization.

FIG. 6 is a graphic representation of the effect of a sense 24mer oligonucleotide corresponding to the same sequence as in FIG. 4(b) on the response of UMR-106 cells to hypotonic stretch (as a control). Cells were loaded with the sense oligonucleotide in parallel with the antisense oligonucleotide used in FIG. 4(b) eighteen hours prior to study during which time the cells were exposed to chronic cyclic strain as described in Example 2 (Methods).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "oligonucleotide" as used herein means a molecule comprised of two or more deoxyribonucleotides, ribonucleotides, analogs or derivatives thereof.

"Oligonucleotide analog" or "oligonucleotide derivative," as those terms are used in conjunction with this invention, refer to moieties that function similarly to deoxyribonucleotides or ribonucleotides, but that have non-naturally occurring portions. Thus, oligonucleotide analogs may have altered sugar moieties or inter-sugar linkages. Exemplary among these are the phosphorothioate and other sulfur-containing species that are known to be useful in this art. They may also contain altered base units or other modifications consistent with the function of oligonucleotides in conjunction with this invention.

In accordance with the invention, it has been demonstrated that an isoform of the voltage-gated CaCh2b channel, an voltage-gated calcium channel protein, is activated by chronic mechanical loading and parathyroid hormone (PTH), two factors known to have anabolic or positive effects on bone formation in osteoblasts. Based on the ability of antisense oligonucleotides to the voltage-gated calcium channel protein to block the increase in whole cell conductance shown to be triggered by the application of chronic, intermittent mechanical strain to osteoblasts, it has further been demonstrated in accordance with this invention that the voltage-gated calcium channel is the signaling mechanism at the molecular level for increasing bone formation in response to mechanical strain.

As a consequence of these and other discoveries described herein, it is believed that application of an antisense strategy against expression of the voltage-gated calcium channel protein in osteoblasts will directly inhibit the abnormally-high bone density formation reflected in the condition known as osteosclerosis. Moreover, these discoveries provide an important analytical tool for and a critical link in the development of methods for the pharmacologic modulation of this channel for clinical treatment of osteoporosis. Thus, it is believed that stimulation of the voltage-gated calcium channel, which serves as a signal transducer for mechanical strain, for example by application of an agonist active in stimulating the expression of the voltage-gated calcium channel protein, will promote the beneficial properties of exercise on bone without the physical exertion that is difficult or impossible for elderly or paralyzed patients or for those unable to enjoy the beneficial effects of strain imposed by Earth's gravity due to extended space flight.

Recent laboratory research also has demonstrated that shear stress, or the mechanical stimulation of the endothelial cells lining the blood vessels by the flow of blood, plays a role in the control of blood pressure. Furthermore, stretching endothelial cells in vitro increases endothelin production and cell proliferation through an increase in intracellular calcium. The increase in intracellular calcium is blocked by gadolinium, a SA-Cat channel inhibitor, suggesting that the SA-Cat channel also transduces the mechanical strain induced by shear stress into cellular biochemical message either to relax or contract the blood vessel. Therefore, modulation of this channel by application of an antisense strategy is also believed to provide the clinician with a means of controlling blood pressure.

These discoveries and their application will be explained in greater detail below.

Mechanical strain increases bone formation and remodeling activity resulting in increased bone mass. Osteoblasts have stretch activated cation (SA-Cat) channels that are

modulated by parathyroid hormone. Chronic, intermittent strain increases the sensitivity of SA-Cat channels to stretch increasing the number of channels open at any given time period and increasing single channel conductance. As a result, an increase in whole cell conductance is demonstrable. Furthermore, spontaneous activity of these channels is observed in chronically strained osteoblasts. These properties have not been previously demonstrated for SA-Cat channels, and they demonstrate that during mechanical loading of the osteoblast, SA-Cat channels are an integral component of the electrical environment and ion flux of the cell.

Chronic, intermittent strain applied to osteoblast-like cells not only affects ion conductance through SA-Cat channels, it also increases gene transcription for the bone matrix proteins. Application of cyclic strain for 24–72 hrs to osteoblast-like cell cultures increases type-1 procollagen message and type-1 collagen secretion. Chronic, intermittent strain also upregulates osteopontin message levels and osteocalcin secretion independent of, but additive to, 1,25 (OH)₂ Vitamin D₃ stimulation.

Calcium channel transcripts in UMR-106 cells were identified using the homology-based reverse transcriptase polymerase chain reaction. Primers were designed from regions that are highly conserved between L-type calcium channels to amplify essentially the entire fourth domain. An 850 base pair clone was isolated, which, upon sequencing, was found to be a CaCh2b variant. In addition, clones corresponding to CaCh1b and CaCh3b variants were isolated. The nucleotide sequence of the cDNA for the CaCh2b clone is set forth in Table 1. This Table depicts the nucleotide sequence of a partial cDNA clone encoding the fourth domain of the α_1 subunit of L-type calcium channel isoforms CaCh1 (ROB1), CaCh2 (ROB2), and CaCh3 (ROB3) isolated from rat osteosarcoma UMR-106 cells. The putative transmembrane domains S1–S6 are indicated by heavy lines under the sequence. The symbol Δ indicates the site of omission of a segment by alternative splicing in the CaCh2b isoform.

Employing an antisense strategy, in accordance with the invention we have demonstrated the ability to inhibit the response of osteoblast-like cells to chronic, cyclic strain. Thus, we have shown that antisense oligonucleotides to the CaCh2 transcript eliminates the biophysical properties of the voltage-gated calcium channel in the cells. Voltage-gated calcium channel activity is eliminated by application of the antisense oligonucleotide.

Antisense therapy is the administration of exogenous oligonucleotides that bind to a target polynucleotide located within the cells. The term "antisense" refers to the fact that such oligonucleotides are complementary to their intracellular targets, e.g., the voltage-gated calcium channel gene or mRNA. See for example, Jack Cohen, *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression*, CRC Press, 1989; and *Synthesis* 1:1–5 (1988). The voltage-gated calcium channel antisense oligonucleotides of the present invention may be RNA or DNA that is complementary to and stably hybridizes with the DNA of, or the mRNA derived from, the voltage-gated calcium gene. Preferably, the antisense oligonucleotides are designed to maximize their specificity for the CaCh2 isoform relative to the CaCh1 and CaCh3 isoforms. Such specificity may be achieved by selecting sequences that

TABLE 1-continued

(SEQ ID NO: 4) (SEQ ID NO: 5)	RO81 RO82	GAAGATCGCC GAAGATTGCC	ATGGTGGACG CTGAATGACA	GGACGCAAAAT CCACAGAGAT	AAACCGGAAC CAATCGGAAC	AACAACCTTCC AACAACCTTCC	AGACTTTTCCC AGACGTTTCCC	ACAAGCTGTC TCAGGGCTGTG	CTGCTGCTCT CTACTGCTCT	TCAGGTGTGC TCAGGTGCGC	700 CACAGGGGCA CACTGGGGAC
(SEQ ID NO: 6)	RO83	GAAGGTTGCC	ATGAGAGATA	ACAAACCAGAT	CAATAGGAAC	AACAACCTTCC	AGACGTTTCCC	CCAGGCAGTG	CTGCTGCTCT	TCAGGTCTGC	AACAGGGGAG
(SEQ ID NO: 4) (SEQ ID NO: 5)	RO81 RO82	GCCTGGCAGG GCTTGGCAGG	AGATCCTGCT ATATCATGCT	GGCCTGCAGC AGCCTGTATG	TACGGGAAAC CCAGGCAAGA	GCTGCGACCC AGTGTGCTCC	GGAGTCTGA. AGAGTCTGAGCTACG CCCAGCAACA	CACCGGGGGA GCACGGAAAG	GGAGTACGCG GGAGACACCC	800 TGTGGCACCA TGTGGCACCA
(SEQ ID NO: 6)	RO83	GCCCGGCAGG	AGATCATGCT	CACCTGCCCTC	CCTGGGAAGC	TGTGTGACCC	GGACTCAGA.TTACA	ACCCAGGACA	GGAATATACT	TGTGGGAGCA
(SEQ ID NO: 4) (SEQ ID NO: 5)	RO81 RO82	ACTTTGCCCTA GTTTCGCTGT	CTACTACTTC CTTCTACTTC	ATCAGCTTCT ATCAGCTTCT	ACATGCTCTG ACATGCTCTG	CGCCTTCCCTG TGCCTTCCCTG	850 CGCCTTCCCTG				
(SEQ ID NO: 6)	RO83	ACTTTGCCAT	TGTTCTACTTC	ATCAGCTTTT	ACATGCTCTG	CGCGTTCCCTG	IVS6				

are not highly conserved between the isoforms. In general, only one or two (e.g., 1 in 20) base pair mismatches in a relatively short oligonucleotide are sufficient to exclude hybridization to non-targeted isoforms. The voltage-gated calcium channel antisense oligonucleotides of the present invention also include derivatives such as S-oligonucleotides (phosphorothioate derivatives or S-oligos) which exhibit voltage-gated calcium channel inhibitory action.

S-oligos (nucleoside phosphorothioates) are isoelectronic analogs of an oligonucleotide (O-oligos) in which a non-bridging oxygen atom of the phosphate group is replaced by a sulfur atom. The S-oligos of the present invention may be prepared by treatment of the corresponding O-oligos with 3H-1,2-benzodithiol-3-one-1,1-dioxide, which is a sulfur transfer reagent. See Iyer, R. P. et al., *J. Org. Chem.* 55:4693-4698 (1990), the disclosures of which are fully incorporated by reference herein. The above substitutions are known in the art to function to enhance the ability of the compositions to penetrate into the region of cells where the RNA whose activity is to be modulated is located. Others, such as alkyl phosphorothioate bonds, N-alkyl phosphoramidates, phosphorodithioates, alkyl phosphonates, and short chain alkyl or cycloalkyl structures may also be useful. Persons with ordinary skill in the art will be able to select other linkages for use in the practice of the invention.

Oligonucleotide analogs may also include species that include at least some modified base forms. Thus, purines and pyrimidines other than those normally found in nature may be so employed. Similarly, modifications on the furanose portions of the nucleotide subunits may also occur as long as the essential tenets of this invention are adhered to. All such analogs are comprehended by this invention so long as they function effectively to hybridize with DNA of or with mRNA derived from the voltage-gated calcium channel gene to inhibit the function of that DNA or mRNA.

The antisense oligonucleotides of this invention preferably comprise about 10 to about 35 bases, preferably from about 15 to 30, and most preferably about 18 to about 26. Two preferred embodiments of the antisense oligonucleotide sequences of the present invention are a 24 mer antisense oligonucleotide having SEQ. ID NO: 2 and a 20 mer antisense oligonucleotide having SEQ. ID NO: 3. These antisense oligonucleotides act just on the 5'-side of the S6 region of the IV domain of the voltage-gated calcium channel α_1 subunit.

The oligonucleotides of this invention are designed to hybridize with DNA or mRNA of the voltage-gated calcium channel. Such hybridization, when accomplished, interferes with the normal function of these DNA/mRNA components. Such inhibited functions may include transcription of the DNA to mRNA, translocation of the RNA to the site of protein translation, and actual translation of protein from the mRNA. The overall effect of the interference caused by the antisense oligonucleotides of the invention is to cause inhibition of the expression of the voltage-gated calcium channel protein. Since it has been established that the voltage-gated calcium channel protein is a component of the signaling mechanism for stretch-induced increased bone formation in osteoblasts, such inhibition works directly to reduce the abnormally-high bone density exhibited in patients suffering from osteosclerosis. Similarly, it is believed that inhibition of voltage-gated calcium channel expression in endothelial tissue by antisense oligonucleotides will inhibit the enhanced cell proliferation and concomitant cellular changes which cause hypertension.

Although use of the antisense strategy is not directly applicable to therapies for treating osteoporosis, antisense oligonucleotides of the present invention represent important analytical tools for studying mechanisms to trigger, rather than inhibit, the expression of voltage-gated calcium channels in osteoblasts to signal enhanced bone matrix formation. Such further investigation using the antisense oligonucleotides as an analytical tool may provide the basis for developing agonists for the voltage-gated calcium channel signaling mechanism that can upregulate the level of bone matrix formation to counteract the loss of bone density caused by osteoporosis.

The present invention is additionally useful in diagnostics and in research in respects other than those specifically referenced above. It is believed that the voltage-gated calcium channels play roles in tissues not yet fully explored. Since the oligonucleotides of this invention hybridize to voltage-gated calcium channels, sandwich and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of oligonucleotides with voltage-gated calcium channels in tissue samples suspected of containing this channel may be accomplished by such means as enzyme conjugation, radiolabelling or other suitable detection systems.

Also included in the present invention are pharmaceutical compositions comprising an effective amount of at least one of the voltage-gated calcium channel antisense oligonucleotides of the invention in combination with a pharmaceutically acceptable carrier. The voltage-gated calcium channel antisense oligonucleotides are preferably coadministered with an agent which enhances the uptake of the antisense molecule by the cells. For example, the voltage-gated calcium channel antisense oligonucleotides may be combined with a lipophilic cationic compound, which may be in the form of liposomes. The use of liposomes to introduce nucleotides into cells is taught, for example, in U.S. Pat. Nos. 4,397,355 and 4,394,448, incorporated herein by reference. Alternatively, the voltage-gated calcium channel antisense oligonucleotides may be combined with a lipophilic carrier such as any one of a number of sterols including cholesterol, cholate and deoxycholic acid. A preferred sterol is cholesterol.

In addition, the voltage-gated calcium channel antisense oligonucleotide may be conjugated to a peptide that is ingested by cells. Examples of useful peptides include peptide hormones or antibodies. By choosing a peptide that is selectively taken up by the neoplastic cells, specific delivery of the antisense agent may be effected. The voltage-gated calcium channel antisense oligonucleotide may be covalently bound via the 5'H group by formation of an activated aminoalkyl derivative. The peptide of choice may then be covalently attached to the activated voltage-gated calcium channel antisense oligonucleotide via an amino and sulfhydryl reactive hetero bifunctional reagent. The latter is bound to a cysteine residue present in the peptide. Upon exposure of cells to the SA-Cat channel antisense oligonucleotide bound to the peptide, the peptidyl antisense agent is endocytosed and the voltage-gated calcium channel antisense oligonucleotide binds to the target voltage-gated calcium channel mRNA to inhibit translation.

The voltage-gated calcium channel antisense oligonucleotides and the pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, or transdermal routes. The dosage administered will be dependent upon the age, health, and weight of

the recipient, type of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For treatment of osteosclerosis, an osteosclerotic-reductive amount is to be administered. For treatment of hypertension, a hypertensive-reductive amount is to be applied.

Compositions within the scope of this invention include all compositions wherein the voltage-gated calcium channel antisense oligonucleotide is contained in an amount that is effective to achieve inhibition of expression of the voltage-gated calcium channel protein sufficient to, when applied to osteoblasts, ameliorate osteosclerosis, and when applied to endothelial tissue, cause a measurably significant reduction in blood pressure, either in localized tissue or system-wide. Although individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art.

In addition to administering the voltage-gated calcium channel antisense oligonucleotides as a raw chemical in solution, the voltage-gated calcium channel antisense oligonucleotides may be administered as part of a pharmaceutically active mixture or preparation containing suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the voltage-gated calcium channel antisense oligonucleotide into preparations that can be used pharmaceutically.

Suitable formulations for parenteral administration include aqueous solutions of the voltage-gated calcium channel antisense oligonucleotides in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

The antisense oligonucleotides of the present invention may be prepared according to any of the methods that are well known to those of ordinary skill in the art. Preferably, the antisense oligonucleotides are prepared by solid phase synthesis. See, Goodchild J. *Bioconjugate Chemistry*, 1:1650167 (1990), for a review of the chemical synthesis of oligonucleotides. Alternatively, the antisense oligonucleotides can be obtained from a number of companies which specialize in the custom synthesis of oligonucleotides.

The following examples illustrate the invention.

EXAMPLE 1

Materials—UMR-106 cells were obtained from American Type Culture Collection (Rockville, Md.). Cell culture media and serum were purchased from Sigma. Sources for other materials were as follows: Fast Track mRNA Isolation Kit (Invitrogen Corp., San Diego, Calif.), GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, Conn.), pBluescript KS⁻ vector and XL-1 Blue bacteria (Stratagene, La Jolla, Calif.), Nuseive, Seakem and Seaplaque agarose (FMC Bioproducts, Rockland, Me.), Sequenase II kit (United States Biochemical Corp., Cleveland, Ohio), DNA ligase (Boehringer Mannheim, Indianapolis, Ind.), acrylamide (Gel-Mix 8, Gibco/BRL, Gaithersburg, Md.), DNA purification kit PZ523 Kit (5 Prime→3 Prime, Inc., Boulder, Colo.), Genescreen Plus nylon membrane (Du Pont NEN Research Products, Boston, Mass.). Labeled probes were prepared using the Riboprobe Gemini System (Promega Corp., Madison, Wis.). [α^{32} P]UTP was purchased from Amersham. Oligonucleotides were synthesized commercially by Macromolecular Resources (Fort Collins, Colo.). Restriction enzymes were purchased from Gibco/BRL.

Cell Culture—UMR-106 cells were cultured in D-MEM/Ham's F-12 medium containing 5% fetal bovine serum. Cells were used between passages 3 and 17.

Purification of RNA—Poly (A⁺) RNA was isolated from cultured cells as described in Bradley, J. E., et al., *Biotechniques* 6, 114–116 (1988) using the FastTrack mRNA Isolation Kit. Briefly, UMR-106 cells were grown to confluence in P-100 culture dishes, washed in phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and harvested by scraping. Cells were pelleted by centrifugation and homogenized with a polytron in the presence of 2% SDS. After incubation with proteinase K, the cell lysate was batch adsorbed to oligo (dT) cellulose and poly (A⁺) RNA was eluted in salt-free buffer. Approximately 20 μ g of poly (A⁺) RNA was isolated from 1 \times 10⁸ cells.

cDNA synthesis and PCR—Poly (A⁺) RNA was reverse transcribed and cDNA was amplified using the GeneAmp RNA PCR kit as follows. Poly (A⁺) RNA, 250 ng, was incubated in a 20 μ l reaction volume containing 2.5 U/ μ l Moloney murine leukemia viral reverse transcriptase, 2.5 μ M random hexamers, 1.0 mM dNTPs and 1 U/ μ l RNase inhibitor in 50 mM KCl, 5 mM MgCl₂, and 10 mM Tris-HCl, pH 8.3, for 10 min at room temperature followed by 15 min at 42° C. and 5 min at 99° C. Subsequently, the samples containing first strand cDNA were brought up to a 100 μ l reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM MgCl₂, 200 μ M of each dNTP, 0.2 μ M of each primer, and 2.5 units of AmpliTaq DNA polymerase and overlaid with 75 μ l of mineral oil. PCR was performed on an automated thermal cycler (Model 480, Perkin-Elmer Cetus) that was programmed to repeat the following cycle 30 times: 60 s at 94° C., 60 s at 60° C. and then 120+2 s/cycle at 72° C.

PCR primers—Oligodeoxynucleotide PCR primers were designed from regions that are highly conserved between L-type calcium channels using the following published sequences: CaCh 1 (rabbit skeletal muscle isoform) Tanabe, T. et al. (1987) *Nature* 328, 313–318, CaCh2 (rat cardiac muscle isoform) Snutch, T. P. et al. (1991) *Neuron* 7, 45–57 and CaCh3 (rat neuroendocrine isoform) Hui, A. et. al. (1991) *Neuron* 7, 35–44. Two pairs of primers were used. One set spanned the entire calcium channel domain IV: upstream primer-1 (UP-1): 5'-gcc ggaaccatcgctcacctccaggagca-3'(SEQ ID NO: 13); and downstream primer (DP-1): 5'-atggaattcgccacraagaggtgatgat-3' (r=a or g)(SEQ ID NO: 14). A second set spanned domain IVS5 through IVS6: upstream primer-2 (UP-2): 5'-gtg ggaattcatcaagtcctccaggccct-3'(SEQ ID NO: 15) and downstream primer-2 (DS-2): 5'-cagg ggateccaagtgtccatgataacage-3'(SEQ ID NO: 16). An additional downstream primer, (DS-3): 5'-ccc gaattcakmgtgttgagcatgatgag-3' (k=t or g, m=a or c)(SEQ ID NO: 17), was designed from sequence in the IVS1 segment of the UMR-106 ROB1 and ROB3 clones to be used with the UP-1 primer to amplify only the 5' end of those clones. Bam H1 or Eco R1 restriction sites (underlined) were included near the 5' ends of the primers to facilitate directional subcloning.

Cloning PCR products—The products of the PCR reaction were directionally cloned into the pBluescript KS⁻ vector. Initially, one half of each PCR reaction was analyzed by electrophoresis on 1% Nuseive/1% Seakem agarose gels. For subcloning, the remaining reaction product was purified by phenol/chloroform/Isoamyl alcohol (25:24:1) extraction and ethanol precipitation, digested with Eco R1 and Bam H1, and isolated as a band from a low melting temperature

1% Seaplaque agarose gel. The vector was also digested with Eco R1 and Bam H1 and isolated on a low melt agarose gel. The ligation reaction was carried out in a 50 μ l volume containing 5 U T4 DNA ligase in 66 mM Tris-HCl, 5 mM MgCl₂, 1 mM dithioerythritol, 1 mM ATP, pH 7.5. Agarose gel slices containing the PCR product or vector were melted at 70° C. for 10 min before the addition of 2–4 μ l to the reaction. The ligation reaction was incubated at 15° C. for 16 h. One tenth of each ligation reaction was used to transform XL-1 Blue bacteria. Competent bacteria were prepared by the calcium chloride method and transformed according to a standard protocol (Sambrook, J., et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., 1989). Transformants were plated onto LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) agar plates containing 50 μ g/ml ampicillin and 12.5 μ g/ml tetracycline that had been coated with 10 μ l of 2% 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside and 40 μ l of 100 mM isopropyl- β -D-thiogalactopyranoside. Individual white colonies were picked and grown overnight in 5 ml of LB medium containing 50 μ g/ml ampicillin at 37° C. Plasmid DNA was prepared from 3.0 ml of overnight culture by alkaline lysis. The presence of an appropriately sized insert was confirmed by Eco R1 and Bam H1 restriction digestion of the plasmid DNA.

Sequencing of Double-Stranded DNA Templates—Plasmid DNA, 5 μ g, was denatured in 200 mM NaOH, 0.2 mM EDTA for 30 min at 37° C., neutralized and precipitated in ethanol. Sequencing was by the dideoxynucleotide chain termination method using the Sequenase II kit. The products were separated by electrophoresis on 8.0% acrylamide gels. Some sequencing was also performed on an automated instrument (Applied Biosystems, Foster City, Calif.) using plasmid DNA purified with the PZ523 Kit. The final sequences were determined from both strands of the cDNA. Sequence analysis was performed using on-line software from the Genetics Computer Group, Inc. (Madison, Wis.).

Northern Blot Analysis—Poly (A+) RNA was electrophoresed on a 0.75% agarose/formaldehyde denaturing gel and electrophoretically transferred to Genescreen Plus nylon membrane. The membranes were UV-crosslinked and baked for 2 h at 80° C. under vacuum. Prehybridization was carried out in 0.25 M NaPO₄, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 50% formamide, 6% SDS at 55° C. for 1 hr. [³²P]-labeled RNA probes were synthesized from linearized cDNA templates using the Riboprobe Gemini System. Hybridization with riboprobes, 2 \times 10⁶ cpm/ml, was under the same conditions as the prehybridization for 20 h. The membranes were washed twice in 0.3M NaCl, 0.03M Na₃Citrate, pH 7.0 (2 \times SSC) for 5 min at room temperature; twice in 0.25M NaPO₄, pH 7.2, 2% SDS, 1 mM EDTA for 30 min at 65° C.; and twice in 0.04 M NaPO₄, pH 7.2, 1% SDS, 1 mM EDTA for 30 min at 65° C. To decrease the background, the membranes were subsequently rinsed three times in 2 \times SSC for 5 min at room temperature, incubated in 2 \times SSC containing 1 μ g/ml RNase A for 15 min at room temperature, and washed twice in 0.04M NaPO₄, pH 7.2, 1% SDS, 1 mM EDTA for 30 min at 65° C. Autoradiography was carried out at –70° C. with an intensifying screen. Hybridization with a riboprobe for human β -actin was used as a positive control.

RESULTS

Homology-based reverse transcriptase-PCR was used to identify transcripts for L-type calcium channels that are present in UMR-106 cells. Primers were designed from regions that are highly conserved between L-type calcium channels to amplify essentially the entire fourth domain (FIG. 1). Three types of cDNA clones were isolated, corre-

sponding to all three of the known L-type calcium channel genes. The CaCh2 isoform (Table 1, ROB2), 850 base pairs, is identical to the rat brain rbc-I transcript (22), except for a 33 base pair (11 amino acids) deletion (FIG. 2) in the S3–S4 linker. This isoform has subsequently been determined (see below) to be a close isoform of the voltage-gated calcium channel gene.

Alternative splicing in the fourth domain of L-type calcium channels can potentially give rise to four variants of CaCh2 (CaCh2a, b, c, d) transcripts (Perez-Reyes, E., et al., *J. Biol. Chem.* 265, 20430–20436 (1990)). This occurs through the use of an alternate splice acceptor site or an exon skipping event in the extracellular IVS3–S4 linker that produces two sizes of transcripts. In addition, mutually exclusive splicing of alternate exons (approximately 50% homologous) encoding the transmembrane IVS3 domain occurs in CaCh2 transcripts (Perez-Reyes, Supra).

According to the Perez-Reyes nomenclature, each of the three L-type CaCh transcripts isolated from UMR-106 cells are of the b type since they contain a specific IVS3 variant and they lack a portion of the IVS3–S4 linker region.

Northern analysis was used to detect expression of mRNA transcripts corresponding to the UMR-106 calcium channel clones (FIG. 3). (Three sizes of mRNAs of approximately 11.7, 8.6, and 4.8 kB were detected.) The ROB2 clone hybridized predominately to the 8.6 kB mRNA, which is the expected size for a CaCh2 isoform transcript. However a larger 12–15 kB transcript detected in other tissues (22,26, 27), including cardiac muscle, aorta, and brain, was not seen. Very high stringency was used during Northern analysis to maximize the specificity of hybridization. Nevertheless, it is possible that the high degree of sequence identity (approximately 76%) between each of the three CaCh isoforms in the region cloned may be responsible for some cross-hybridization as a result of the relatively high abundance of those transcripts.

EXAMPLE 2

METHODS

Cell culture: UMR-106.01 cells (passages 12–18) were grown in Minimal Essential Medium with Eagles modification, non-essential amino acids and Earle's salts (Sigma, St. Louis, Mo.) supplemented with 10% fetal bovine serum (Gibco, Grand Island, N.Y.). Cells were plated onto flexible, type I collagen-coated, silicone-bottomed 6 well culture plates (Flexcell, Inc., McKeesport, Pa.), fed twice weekly and maintained in a humidified atmosphere of 95% air/5% CO₂ at 37° C. When the cells were approx. 75% confluent, they were transfected with either the sense or antisense oligodeoxynucleotides (oligonucleotide) as described below. Culture plates were placed on the Flexercell apparatus which uses vacuum to stretch the silicone bottoms and cyclic stretch was applied for 12–30 hrs at 3 cycles/min. A maximal strain of 12% displacement was applied at the edge of the culture plate falling to 0% displacement at the center.

Transfection of oligonucleotide's. Two antisense oligonucleotides (24-mer and 20-mer)(respectively, SEQ ID NO: 2 and NO: 3) and a sense (24-mer) oligonucleotide were developed from the sequence of a cDNA clone of the α_1 subunit of an L-type calcium channel (CaCh2) isolated from UMR-106 by reverse transcriptase-polymerase chain reaction (SEQ ID NO:1). The antisense oligonucleotides were commercially synthesized by Macromolecular Resources (Fort Collins, Colo.). The antisense oligonucleotides were introduced into the UMR cells using streptolysin O (Sigma, St. Louis, Mo.) permeabilization. After the UMR cells had

been plated onto the flexible, silicone-bottomed culture plates and grown to 75% confluency, the medium was removed and the cells were washed with a permeabilization buffer consisting of (in mM): 137 NaCl, 5.6 glucose, 2.7 KCl, 2.7 EGTA, 1 Na-ATP, 100 PIPES and 0.1% bovine serum albumin, pH 7.4. The permeabilization buffer containing 0.5 U/ml streptolysin O and 100 μM of the appropriate oligonucleotide was then placed on the cells for 5 min at room temperature. This solution was then removed and the normal medium with 10% fetal bovine serum was added to the cells. For control experiments, UMR cells were permeabilized with the same concentration of streptolysin O with no oligonucleotide present.

Patch Clamp Studies. Following application of chronic, intermittent strain, the silicone bottom of the cluster was removed and transferred to a recording chamber (1 ml total volume (Biophysics Technologies, Baltimore, Md.) which was modified to permit rapid exchange of the bathing solution with minimal perturbation to the cells. Cells were bathed in a mammalian Na⁺ Ringer's solution consisting of (in mM): 136 NaCl; 5.5 KCl; 1 MgCl₂; 1 CaCl₂; and 20 N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid] (HEPES) buffer; titrated to pH 7.3 with NaOH. The strain pattern associated with the Flexcell apparatus is non-uniform (1). The profiles of strain range from 120,000 μE at the edge of the well (12% maximal displacement) to 0 μE at the center. The cells used in this study were subjected to similar magnitudes of strain since patches were always performed in an area 10mm from the edge of the well. Strain applied to this area estimated at 10,000 to 20,000 μE using the strain curve described by Banes, et al. Comparisons were made between chronically stretched control and oligonucleotide-treated cells from the same passage number and at the same level of confluency. To impose membrane strain on the UMR cells during the patch clamp studies, 10 ml 65 mM NaCl (182 mOsm) hypotonic Ringer's solution was perfused into the chamber.

To measure membrane potentials and whole cell conductances, the nystatin performed patch technique was used under current clamp conditions. The pipette solution in these experiments consisted of (in mM): 12 NaCl; 64 KCl; 28 K₂SO₄; 47 sucrose; 1 MgCl₂; 0.5 EGTA; 20 HEPES; titrated to 7.35 with KOH. Nystatin was added at a concen-

tration of 300 μg/ml to permeabilize the patch. Access resistances of <40 MΩ were consistently achieved with this concentration of nystatin. Whole cell conductance measurements were made by pulsing ±50 pA across the membrane.

RESULTS

We examined the effects of the antisense and sense oligonucleotides on the strain-induced increase in whole cell conductance in UMR-106.01 cells, which we have shown to be the result of upregulation of the stretch activated cation channel. A previous report had demonstrated that antisense oligonucleotides produce a time dependent inhibition of the chlorothiazide-induced increase in [CaU²⁺]_i in the mouse distal convoluted tubule cells. This inhibition starts 6 hrs after introduction of the antisense and peaks at 18 hrs. The delay in inhibition was attributed to the turnover of existing proteins prior to the inhibition of new synthesis by the antisense. Cell conductance measurements of the strained UMR cells which had been transfected with the 24-mer antisense found a similar time dependence which began at 12 hr. Measurements at 18 hr found 100% inhibition of the strain-induced increase in whole cell conductance (FIGS. 4(a), 4(b)). Parallel time control cells which had been sham permeabilized exhibited no decrease in whole cell conductance. A similar time course and inhibition was observed for the 20 mer antisense (FIGS. 5(A), 5(B)). Transfection of the sense oligonucleotide produced no significant changes in the whole cell conductance increases associated with hypotonic swelling (FIG. 6). Return of the whole cell conductance response to hypotonicity was observed 26 hr following introduction of the antisense oligonucleotides. This response was significantly higher than the control response, suggesting some feedback mechanism on the gene to increase expression of this protein.

In view of the above, it will be seen that the several objects of the invention are achieved and other advantageous results attained.

As various changes could be made in the above compositions and methods without departing from the scope of the invention, it is intended that all matter contained in the above description shall be interpreted as illustrative and not in a limiting sense.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 17

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7860 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCAACACCT TAATATTTAT ATCTCCACGT TGTTTTATTT TTTTCCCCT TGTTTTGGGG	6 0
GGCTTTTATT TTCCTTTTGT TTTTAAAATT TTATCCTTGT ATATCACAAT AATGGAAAGA	1 2 0
AAGTTTATAG TGTCTTTCAC AAAGGAGCGT AGTTTAAATG CCCCGCCGCG CCCGCGCCCG	1 8 0
CCCCTGCCAG AACGGCGCTC GGCGGCGCGG CCCGGAGCGG CGGCGGCGGT GGCGGCGGGC	2 4 0

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GCGGCCGTTT	CCGCGGGGCTC	GCCCTCAGGT	GTTGCGGGCT	GCCGTCGCCG	AAGATCGCGG	300
GTCGGGGCCT	CGCGGGCGATC	GCCCTGGGCG	GGCCGGAGAC	GCCTCGGGCC	CCCTGGCGGC	360
TCGGGGTCCA	CCCGGCGCCG	CGGGCCCCGCC	GCTCTCCCTC	GCCTCGCCTT	TGCGCCTCTT	420
CTCGCTCTGC	CTCTCCATTT	ATTATTATTA	TCATTTTTGT	TTTCAAATGG	TGTAGCCGCC	480
AGAGGTGCGG	TGCTAAATTC	TTGGAAGGGG	CCCGGATGTA	CTGAGGATGC	ATTACAATCT	540
CACGAAAGGA	GGCGGTAGTG	GAAAGCAGCA	GTTTTTGGTG	TTTGGTGCAA	TAATGGGGAT	600
CAGGTAATCA	CCCGAAGGAG	CAAGAACCAC	TGCGGATCCA	CGGCTTCCTG	GATTTGCGCG	660
AGAGCCGCCG	GCCTCGGAGG	AGGGATCCAT	CCAGAGGCTC	GCGGGCTGTT	GCTGCATTTT	720
TTCTCTTTTG	TGGCTTCTCC	TTTCCAAGCA	GTTTTTGGCC	AATGGTCAAT	GAAAAACACGA	780
GGATGTACGT	TCCAGAGGAA	AACCACCAAG	GTTCCAACATA	TGGGAGCCCA	CGCCAGCTC	840
ATGCCAACAT	GAATGCCAAT	GCAGCTGCAG	GACTTGCCCC	CGAGCACATC	CCTACTCCAG	900
GGGCAGCACT	GTCCTGGCAG	GCAGCCATCG	ATGCCGCCCG	GCAGGCCAAG	TTAATGGGCA	960
GTGCTGGCAA	TGCGACCATC	TCTACCGTCA	GTTCCACACA	GCGGAAGCGG	CAGCAGTATG	1020
GGAAACCCAA	GAAGCAGGGG	GGCACAACTG	CCACACGGCC	GCCCCGGGCT	CTGCTCTGCC	1080
TGACTCTGAA	GAACCCCATC	AGGAGGGCAT	GCATCAGCAT	TGTTGAATGG	AAACCATTTG	1140
AAATAATTAT	TTTATTGACG	ATTTTTGCCA	ATTGTGTGGC	CTTAGCAATC	TATATTCCCT	1200
TTCCGGAAGA	TGACTCCAAC	GCCACCAACT	CCAACCTGGA	ACGGGTGGAG	TATCTCTTCC	1260
TCATCATTTT	TACCGTGGA	GCATTTTTAA	AAGTGATTGC	CTACGGACTA	CTCTTCCACC	1320
CCAACGCTTA	CCTCCGCAAC	GGTTGGAATT	TACTAGATTT	TATAATCGTG	GTTGTAGGGC	1380
TTTTTAGTGC	AATTTTAGAA	CAAGCAACCA	AAGCTGACGG	GGCCAACGCT	CTGGGAGGGA	1440
AAGGAGCGGG	ATTCGATGTG	AAGGCACTGA	GAGCTTTCCG	TGTGCTTCGT	CCCCTGCGGC	1500
TGGTGTCTGG	AGTCCCAAGT	CTCCAGGTGG	TCCTGAACTC	CATCATCAAG	GCCATGGTGC	1560
CCCTGCTGCA	CATTGCCCTC	CTCGTGCTCT	TCGTCATCAT	CATCTATGCC	ATTATCGGCC	1620
TGGAGCTCTT	CATGGGGAAG	ATGCACAAGA	CCTGCTACAA	CCAGGAGGGC	ATAATAGATG	1680
TTCCAGCGGA	AGAGGATCCC	TCCCCTTGTT	CTTTGGAGAC	AGGCCATGGG	CGACAGTGTC	1740
AGAACGGGAC	CGTGTGCAAG	CCCGGGTGGG	ATGGGCCCCA	GCACGGCATC	ACCAACTTCG	1800
ACAAC TTCG	CTTCGCCATG	CTGACGGTGT	TCCAGTGTAT	CACCATGGAG	GGCTGGACAG	1860
ACGTGCTGTA	CTGGATGCAA	GACGCTATGG	GCTATGAGTT	GCCCTGGGTG	TATTTTGTCA	1920
GTCTGGTCAT	CTTTGGATCC	TTTTTTCGTT	TAAATCTGGT	TCTCGGTGTT	TTGAGCGGAG	1980
AGTTTTCCAA	AGAGAGGGAG	AAAGCCAAAAG	CTCGAGGAGA	CTTCCAGAAG	CTTCGTGAGA	2040
AGCAGCAGCT	AGAAGAAGAT	CTCAAAGGCT	ACCTGGACTG	GATCACCCAG	GCGGAAGACA	2100
TAGACCCTGA	GAATGAGGAC	GAGGGCATGG	ATGAAGACAA	ACCCCGAAAC	ATGAGCATGC	2160
CCACAAGTGA	GAATGAGTCT	GTCAACACCG	AAAACGTGGC	TGGAGGTGAC	ATCGAGGGTG	2220
AAAAC TGTG	AGCCCCGGCTT	GCCCACCGGA	TCTCCAAATC	CAAGTTCAGC	CGCTACTGGC	2280
GCCGGTGGAA	TAGATTCTGC	AGAAGAAAGT	GCCGTGCCGC	AGTTAAGTCC	AACGTCTTCT	2340
ACTGGCTGGT	GATCTTCCCTG	GTGTTCCCTCA	ACACCCTCAC	CATTGCCTCC	GAACACTACA	2400
ACCAGCCTCA	TTGGCTCACA	GAAGTGCAAG	ACACGGCCAA	CAAGGCCCTC	CTGGCCCTTT	2460
TCACGGCAGA	AATGCTCCTG	AAGATGTACA	GCCTGGGTCT	GCAGGCCTAC	TTTGTATCCC	2520
TCTTCAACCG	CTTTGACTGT	TTCATTGTGT	GCGGGGGCAT	CCTGGAGACC	ATCCTGGTGG	2580
AGACGAAGAT	CATGTCGCCC	CTGGGCATCT	CCGTGCTGAG	ATGTGTGCGG	CTGCTCAGGA	2640

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TTTTCAAGAT	CACCAGGTAC	TGGAACCTCCC	TGAGCAACCT	CGTGGCGTCC	TTGCTGAACT	2700
CAGTGCGCTC	CATCGCCTCC	CTGCTGCTAC	TCCTCTTCCT	CTTCATCATC	ATCTTCTCCC	2760
TCCTGGGGAT	GCAGCTGTTT	GGTGGAAAGT	TCAACTTTGA	TGAGATGCAG	ACCCGTAGGA	2820
GCACGTTTGA	TAACCTCCCC	CAGTCTCTCC	TCACTGTGTT	TCAGATCCTG	ACCGGGGAGG	2880
ACTGGAATTC	GGTGATGTAT	GATGGGATCA	TGGCTTATGG	CGGCCCTCT	TTTCCAGGGA	2940
TGTTAGTCTG	TATTTACTTC	ATCATCCTCT	TCATCTGTGG	AAATTATATC	CTACTGAATG	3000
TGTTCTTGGC	CATTGCGGTG	GACAACCTGG	CTGATGCGGA	GAGCCTGACC	TCTGCCCCAA	3060
AAGAGGAAGA	AGAAGAGAAG	GAGAGAAAAG	AGCTGGCCAG	GACTGCCAGC	CCAGAAAAAG	3120
AACAGGAGGT	GATGGAGAAA	CCAGCAGTAG	AGGAGAGCAA	AGAGGAGAAA	ATTGAGCTGA	3180
AGTCCATTAC	AGCAGATGGA	GAATCCCCGC	CCACTACCAA	GATCAACATG	GATGACCTCC	3240
AGCCAGTGA	AAATGAGGAC	AAGAGTCCCC	ACTCCAACCC	AGACACTGCA	GGTGAAGAGG	3300
ATGAGGAGGA	GCCCGAGATG	CCTGTGGGCC	CACGCCCCCG	GCCCCTGTCT	GAGCTGCACC	3360
TTAAGGAAAA	GGCAGTCCCC	ATGCCGGAAG	CCAGTGCATT	TTTCATCTTC	AGCCCCAAAC	3420
ACAGGTTCCG	CCTGCAGTGC	CACCGCATTG	TCAATGACAC	GATCTTCACC	AACCTCATCC	3480
TCTTCTTCAT	TCTGCTCAGT	AGCATCTCCC	TGGCGGCTGA	GGACCCCGTC	CAGCACACCT	3540
CCTTCAGGAA	CCACATCCTA	GGCAATGCAG	ACTATGTCTT	CACTAGTATC	TTTACATTAG	3600
AAATTATCCT	TAAGATGACT	GCTTACGGGG	CTTTCCTGCA	CAAGGGCTCT	TTCTGCCGAA	3660
ATTACTTCAA	TATCCTGGAC	CTGCTGGTGG	TTAGCGTGTC	CCTCATCTCC	TTTGGCATTG	3720
AGTCCAGCGC	GATCAACGTT	GTGAAGATTT	TAAGAGTGCT	TCGTGTCCTC	AGGCCCCCTG	3780
GGGCCATCAA	CAGGGCCAAG	GGGCTAAAGC	ACGTCGTTCA	GTGTGTGTTT	GTGGCCATCC	3840
GGACCATTGG	AAACATTGTA	ATTGTCACCA	CTCTGCTGCA	GTTTCATGTT	GCCTGCATTG	3900
GGGTCCAGCT	CTTCAAGGGA	AAGCTCTATA	CCTGTTCCGA	TAGTTCCAAA	CAGACGGAGG	3960
CAGAATGCAA	GGGTAACAT	ATAACATACA	AAGACGGAGA	AGTTGACCAC	CCCATTATCC	4020
AGCCTCGAAG	TTGGGAGAAC	AGCAAGTTCG	ACTTTGACAA	TGTTCTGGCA	GCCATGATGG	4080
CCCTCTTTAC	CGTCTCCACC	TTCGAGGGGT	GGCCAGAGCT	GCTGTACCGC	TCCATTGACT	4140
CCCACACAGA	AGACAAGGGT	CCCATCTACA	ACTATCGTGT	GGAGATCTCC	ATCTTCTTCA	4200
TCATCTACAT	CATCATCATT	GCCTTCTTCA	TGATGAACAT	CTTCGTGGGT	TTCGTCAATTG	4260
TCACCTTCCA	GGAGCAGGGG	GAACAAGAAT	ACAAGAACTG	TGAGCTGGAC	AAGAACCAGA	4320
GACAATGTGT	GGAATATGCC	CTCAAGGCCC	GACCCTTGCG	AAGGTACATC	CCCAAGAACC	4380
AGCACCAGTA	CAAAGTGTGG	TACGTGGTCA	ACTCCACCTA	CTTCGAGTAT	CTGATGTTTCG	4440
TCCTCATCCT	GCTCAACACC	ATCTGCCTGG	CCATGCAGCA	CTACGGCCAG	AGCTGCCTCT	4500
TCAAAATCGC	CATGAATATA	CTCAACATGC	TTTTCACTGG	CCTCTTCACG	GTGGAGATGA	4560
TCCTGAAGCT	CATTGCCTTC	AAACCCAAGG	GTTACTTTAG	TGATCCCTGG	AATGTTTTTG	4620
ACTTCCTCAT	CGTCATTGGG	AGCATAATTG	ATGTCATTCT	CAGTGAAACT	AATCCAGCTG	4680
AACATACCCA	ATGCTCTCCC	TCTATGAGTG	CAGAGGAGAA	CTCCCGCATC	TCCATCACCT	4740
TCTTCCGCCT	CTTCCGGGTC	ATGCGCCTGG	TGAAGCTGCT	GAGCCGAGGG	GAAGGCATCC	4800
GGACCCTGCT	GTGGACCTTC	ATCAAGTCCT	TCCAGGCCCT	GCCCTATGTG	GCCCTTTTGA	4860
TTGTGATGCT	GTTCTTTATC	TATGCAGTGA	TTGGGATGCA	GGTATTTGGG	AAGATTGCCC	4920
TGAATGACAC	CACAGAGATC	AATCGGAACA	ACAACCTTCCA	GACGTTCCCT	CAGGCTGTGC	4980
TACTGCTCTT	CAGGTGCGCC	ACTGGGGAGG	CCTGGCAGGA	TATCATGCTA	GCCTGTATGC	5040

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CAGGCAAGAA	GTGTGCTCCA	GAGTCTGAGC	CCAGCAACAG	CACGGAAGGG	GAGACACCCT	5100
GTGGCAGCAG	TTTCGCTGTC	TTCTACTTCA	TCAGCTTCTA	CATGCTCTGT	GCCTTCCTGA	5160
TCATCAACCT	CTTTGTAGCT	GTTATCATGG	ACAACTTTGA	CTACCTGACT	AGGGATTGGT	5220
CTATCCTTGG	TCCCCATCAC	CTGGATGAAT	TCAAGAGAAT	CTGGGCCGAA	TATGACCCTG	5280
AAGCCAAGGG	TCGGATCAAA	CAC TTGGATG	TGGTGACCCT	CCTCCGTCGA	ATT CAGCCCC	5340
CACTGGGTTT	TGGGAAGTTG	TGTCCTCACC	GTGTGGCCTG	CAAACGCCTG	GTGTCCATGA	5400
ACATGCCTCT	GAACAGTGAT	GGGACGGTCA	TGTTCAATGC	TACACTGTTT	GCCCTTGTC A	5460
GGACAGCCCT	GAGGATCAAA	ACAGAAGGGA	ACCTAGAGCA	AGCCAATGAG	GAGCTGAGAG	5520
CCATCATCAA	GAAAA TCTGG	AAGAGGACCA	GCATGAAGCT	GTTGGACCAG	GTGGTGCCCC	5580
CTGCAGGTGA	TGACGAGGTC	ACAGTGGGCA	AGTTCTATGC	CACCTTCCTG	ATCCAAGAGT	5640
ACTTCAGGAA	ATTCAAGAAG	CGAAAAGAGC	AGGGGCTGGT	CGGCAAGCCC	TCGCAGAGGA	5700
ATGCACTGTC	TCTGCAGGCT	GGCTTACGCA	CCTTG CATGA	CATTGGGCCT	GAGATCCGGA	5760
GAGCCATCTC	TGGGGATCTG	ACGGCTGAGG	AGGAGCTGGA	CAAGGCTATG	AAGGAGGCAG	5820
TGTCTGCTGC	CTCCGAAGAC	GACATCTTCA	GGAGGGCTGG	AGGCCTGTTT	GGCAACCACG	5880
TCAGCTACTA	CCAGAGTGAC	AGCAGGAGCA	ACTTCCCTCA	GACGTTTGCC	ACCCAACGCC	5940
CACTGCACAT	CAACAAGACA	GGGAACAACC	AAGCGGACAC	CGAATCACCG	TCCCATGAGA	6000
AGCTGGTGGA	CTCCACTTTT	ACCCCCAGCA	GCTACTCATC	CACGGGCTCC	AATGCCAACA	6060
TCAACAATGC	CAACAACACT	GCCCTGGGCC	GCTTCCCCCA	CCCTGCTGGC	TACTCCAGCA	6120
CGGTCAGCAC	TGTGGAGGGC	CATGGGCCTC	CCTTG TCCCC	TGCTGTCCGG	GTACAGGAGG	6180
CAGCATGGAA	ACTCAGCTCT	AAGAGGTGCC	ACTCCCGAGA	GAGCCAGGGG	GCCACGGTGA	6240
GTCAGGATAT	GTTTCCAGAT	GAGACCCGCA	GCAGCGTGAG	GCTGAGCGAA	GAAGTTGAGT	6300
ACTGCAGTGA	GCCCAGCCTG	CTCTCCACAG	ATATACTCTC	CTACCAGGAC	GATGAAAACC	6360
GACAACTGAC	CTGTCTAGAG	GAGGACAAGA	GGGAGATCCA	GCCATCTCCG	AAGAGGAGTT	6420
TCCTTCGCTC	TGCCTCTCTA	GGTCGAAGGG	CCTCCTTCCA	TCTGGAATGT	CTAAAGCGAC	6480
AAAAGGATCA	AGGAGGAGAC	ATCTCTCAGA	AGACAGCCTT	GCCCTTG CAT	CTGGTT CATC	6540
ACCAGGCATT	GGCAGTG GCA	GGCTTGAGCC	CCCTCCTGCA	GAGAAGCCAT	TCTCCTTCCA	6600
CGTTTCCCAG	GCCACGCCCC	ACGCCCCCTG	TCACTCCAGG	CAGCCGGGGC	AGGCCCCTAC	6660
AGCCCATCCC	TACCCTGCGG	CTGGAGGGGG	CGGAGTCCAG	TGAGAAACTC	AACAGCAGCT	6720
TCCCGTCCAT	CCACTGCAGC	TCCTGGTCAG	AGGAGACCAC	AGCCTGTAGT	GGGGGCAGCA	6780
GCATGGCCCG	GAGAGCCCGG	CCCGTCTCCC	TCACCGTGCC	CAGCCAGGCT	GGAGCTCCAG	6840
GCAGACAGTT	CCACGGCAGC	GCCAGCAGCC	TGGTGGAAGC	GGTCTTGATT	TCAGAAGGAC	6900
TGGGACAGTT	TGCTCAAGAT	CCCAAGTTCA	TCGAGGTCAC	CACACAGGAG	CTGGCTGACG	6960
CCTGCGATAT	GACAATAGAG	GAGATGGAGA	ACGCCGCAGA	CAACATCCTC	AGCGGGGGCG	7020
CCCAGCAGAG	CCCCAACGGC	ACCCTCTTAC	CTTTTG TGAA	CTGCAGGGAC	CCAGGGCAGG	7080
ACAGGGCTGT	GGTCCCAGAG	GACGAGAGCT	GTGTATATGC	CCTGGGGCGA	GGCCAGAGCG	7140
AGGAAGCGCT	CCCGGACAGC	AGGTCCTATG	TCAGCAACCT	G TAGTCCGCA	GGGCTGGCGA	7200
GACGCGGGTG	TTTTTTATT C	GTTTCAATGT	TCCTAATGGG	TTCGTTTCAG	AAGTGCCTCA	7260
CTGTTCTCGT	GACCTGGAGG	TAACCGGAAC	AGCGTCTTCA	T TCACTGCTG	TCGGGATAAG	7320
CCTCAGAGCT	GGGCGGTGTA	CGAAGTCGGC	TTTTCAGGGG	AGAAGGCCAA	GGCCGTGGTG	7380
CGGGGGCTCC	AGCACCTTCC	CACGGCAGCA	CCGCCCAAAG	GACCCCACCC	CCCCTAAGCA	7440

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AAAGGGTGTT	TTCCCCTTGC	TTGTATAAAC	AGTCATTTGC	ACATGTTCTG	TCTGAGCCTG	7500
GCCGTCTCTA	TGGAGCAGGG	CCCCAGGGAT	CTATGGCAGG	AATGGCCAGT	GTCCCCAGTA	7560
GGAGCCGGAA	GGTGGCTGCA	AGGTTCCCAG	CAGTGCAGAT	CTGGTCCCTA	TTGCCCTTCA	7620
GGGACCCTTC	CCCTGCTGGA	ACTGAGGAGC	AGGTGCAGGA	GCCAGTGCAG	ACCACACCAC	7680
CTGCCCTCAG	CTAGCCAGAC	CTGGGGGCGC	AGGCTGCTTG	CCTGGTGCTC	TGGGTTTCAT	7740
AGTTTGATGG	TTCTTGTCAG	CATGTTGCGG	TTTTCTAGGT	TTTGATTTCT	TTATTACTAT	7800
TTGTTGTGTT	TTCCCACGGG	GAGGGGAGGA	AGAAGAGCGT	TTACAAC TGC	GCAGCTCACG	7860

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTTCCGTGC	TGTTGCTGGG	CTCA	24
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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACTCTGGAGC	ACACTTCTTG	20
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(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 844 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGGGAGACT	GAGTATAAGA	ACTGTGAGCT	GGACAAGAAC	CAGCGCCAAT	GTGTGCAGTA	60
TGCCCTGAAG	GCCCGCCCGC	TGAGGTGTTA	CATCCCCAAA	AACCCATACC	AGTATCAGGT	120
GTGGTATGTC	GTCAC TTCCT	CCTACTTTGA	ATACCTGATG	TTTGCTCTCA	TCATGCTCAA	180
CACTATCTGC	CTAGGCATGC	AGCATTACAA	CCAGTCGGAA	CAGATGAACC	ACATCTCGGA	240
CATCCTCAAT	GTGGCTTTCA	CCATCATCTT	CACCCTGGAG	ATGATCCTCA	AGCTCATAGC	300
TTTCAAGCCC	AGGGGCTATT	TTGGAGACCC	CTGGAACGTG	TTTGACTTTC	TAATCGTCAT	360
TGGCAGCATC	ATTGACGTTA	TCCTGAGTGA	GATCGATGAC	CCAGATGAGA	GCGCCCGCAT	420
CTCCAGTGCC	TTCTTCCGCC	TGTTCCGAGT	CATGCGACTG	ATCAAGCTGC	TGAGCCGGGC	480
GGAGGGTGTG	CGCACCCCTGC	TCTGGACGTT	CACCAAGTCC	TTCCAGGCCC	TGCCGTATGT	540
GGCTTTGCTG	ATCGTCATGC	TCTTCTTCAT	CTACGTGTGC	ATCGGCATGC	AGATGTTTCGG	600
GAAGATCGCC	ATGGTGGACG	GGACGCAAAT	AAACCGGAAC	AACAAC TTCC	AGACTTTCCC	660
ACAAGCTGTG	CTGCTGCTCT	TCAGGTGTGC	CACAGGGGCA	GCCTGGCAGG	AGATCCTGCT	720
GGCCTGCAGC	TACGGGAAAC	GCTGCGACCC	GGAGTCTGAC	TACGCACCGG	GGGAGGAGTA	780
CGCGTGTGGC	ACCAACTTTG	CCTACTACTA	CTTCATCAGC	TTCTACATGC	TCTGCGCCTT	840

CCTG 844

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 850 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGGAACAA	GAATACAAGA	ACTGTGAGCT	GGACAAAGAAC	CAGAGACAAT	GTGTGGAATA	60
TGCCCTCAAG	GCCCGACCCT	TGCGAAGGTA	CATCCCCAAG	AACCAGCACC	AGTACAAAGT	120
GTGGTACGTG	GTCAACTCCA	CCTACTTCGA	GTATCTGATG	TTCGTCCTCA	TCCTGCTCAA	180
CACCATCTGC	CTGGCCATGC	AGCACTACGG	CCAGAGCTGC	CTCTTCAAAA	TCGCCATGAA	240
TATACTCAAC	ATGCTTTTCA	CTGGCCTCTT	CACGGTGGAG	ATGATCCTGA	AGCTCATTGC	300
CTTCAAACCC	AAGGGTTACT	TTAGTGATCC	CTGGAATGTT	TTTGACTTCC	TCATCGTCAT	360
TGGGAGCATA	ATTGATGTCA	TTCTCAGTGA	AACTAATAGT	GCAGAGGAGA	ACTCCCGCAT	420
CTCCATCACC	TTCTTCCGCC	TCTTCCGGGT	CATGCGCCTG	GTGAAGCTGC	TGAGCCGAGG	480
GGAAGGCATC	CGGACCCTGC	TGTGGACCTT	CATCAAAGTCC	TTCCAGGCCC	TGCCCTATGT	540
GGCCCTTTTG	ATTGTGATGC	TGTTCTTTAT	CTATGCAGTG	ATTGGGATGC	AGGTATTTGG	600
GAAGATTGCC	CTGAATGACA	CCACAGAGAT	CAATCGGAAC	AACAACCTTC	AGACGTTCCC	660
TCAGGCTGTG	CTACTGCTCT	TCAGGTGCGC	CACTGGGGAG	GCCTGGCAGG	ATATCATGCT	720
AGCCTGTATG	CCAGGCAAGA	AGTGTGCTCC	AGAGTCTGAG	CCCAGCAACA	GCACGGAAGG	780
GGAGACACCC	TGTGGCAGCA	GTTTCGCTGT	CTTCTACTTC	ATCAGCTTCT	ACATGCTCTG	840
TGCCTTCCTG						850

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 844 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGAGAAAAG	GAGTATAAGA	ACTGTGAGCT	GGACAAAAAT	CAGCGTCAGT	GTGTGGAATA	60
TGCCTTGAAG	GCCCGCCCCCT	TAAGGAGATA	CATCCCCAAA	AACCCATACC	AGTACAAGTT	120
CTGGTACGTG	GTGAACTCCT	CGCCTTTCGA	ATATATGATG	TTTGTCTCTCA	TCATGCTCAA	180
CACGCTCTGC	CTGGCCATGC	AGCACTATGA	GCAATCCAAG	ATGTTCAATG	ACGCCATGGA	240
CATTCTGAAC	ATGGTCTTCA	CGGGGGTCTT	CACCGTTGAG	ATGGTTTTGA	AAGTCATCGC	300
ATTTAAGCCC	AAGGGGTATT	TTAGTGACGC	CTGGAACACG	TTTGACTCCC	TCATCGTAAT	360
CGGCAGCATT	ATAGACGTGG	CACTCAGCGA	AGCTGACAAC	TCTGAAGAGA	GCAATAGAAT	420
CTCCATCACC	TTTTTCCGTC	TTTTCCGAGT	GATGCGGTTG	GTGAAGCTTC	TCAGCAGAGG	480
GGAAGGCATC	CGGACTCTGC	TATGGACCTT	CATTAAGTCC	TTCCAGGCAC	TCCCATATGT	540
CGCCCTCCTC	ATTGCCATGC	TCTTCTTCAT	CTACGCCGTC	ATTGGCATGC	AGATGTTTGG	600
GAAGGTTGCC	ATGAGAGATA	ACAACCAGAT	CAATAGGAAC	AACAACCTTC	AGACGTTTCC	660
CCAGGCAGTG	CTGCTGCTCT	TCAGGTGTGC	AACAGGGGAG	GCCCGGCAGG	AGATCATGCT	720
CGCCTGCCTC	CCTGGGAAGC	TGTGTGACCC	GGACTCAGAT	TACAACCCAG	GAGAGGAATA	780

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TACTTGTGGG AGCAACTTTG CCATTGTCTA CTTTCATCAGC TTTTACATGC TCTGCGCGTT	840
CCTG	844

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 amino acid residues
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

P r o	T r p	A s n	V a l	P h e	A s p	P h e	L e u	I l e	V a l	I l e	G l y	S e r	I l e	I l e	A s p
1				5					10					15	
V a l	I l e	L e u	S e r	G l u	I l e	A s p	A s p	P r o	A s p	G l u	S e r	A l a	A r g	I l e	S e r
			20					25					30		
S e r	A l a	P h e	P h e	A r g	L e u	P h e	A r g	V a l	M e t	A r g	L e u	I l e	L y s	L e u	L e u
		35					40					45			
S e r	A r g	A l a													
		50													

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 70 amino acid residues
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

P r o	T r p	A s n	V a l	P h e	A s p	P h e	L e u	I l e	V a l	I l e	G l y	S e r	I l e	I l e	A s p
1				5					10					15	
V a l	I l e	L e u	S e r	G l u	I l e	A s p	T h r	P h e	L e u	A l a	S e r	S e r	G l y	G l y	L e u
			20					25					30		
T y r	C y s	L e u	G l y	G l y	G l y	C y s	G l y	A s n	V a l	A s p	P r o	A s p	G l u	S e r	A l a
		35					40					45			
A r g	I l e	S e r	S e r	A l a	P h e	P h e	A r g	L e u	P h e	A r g	V a l	M e t	A r g	L e u	I l e
	50					55					60				
L y s	L e u	L e u	S e r	A r g	A l a										
65					70										

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 amino acid residues
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

P r o	T r p	A s n	V a l	P h e	A s p	P h e	L e u	I l e	V a l	I l e	G l y	S e r	I l e	I l e	A s p
1				5					10					15	
V a l	I l e	L e u	S e r	G l u	T h r	A s n	S e r	A l a	G l u	G l u	A s n	S e r	A r g	I l e	S e r
			20					25					30		
I l e	T h r	P h e	P h e	A r g	L e u	P h e	A r g	V a l	M e t	A r g	L e u	V a l	L y s	L e u	L e u
		35					40					45			
S e r	A r g	G l y													
		50													

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 62 amino acid residues
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Pro Trp Asn Val Phe Asp Phe Leu Ile Val Ile Gly Ser Ile Ile Asp
1 5 10 15
Val Ile Leu Ser Glu Thr Asn Pro Ala Glu His Thr Gln Cys Ser Pro
20 25 30
Ser Met Ser Ala Glu Glu Asn Ser Arg Ile Ser Ile Thr Phe Phe Arg
35 40 45
Leu Phe Arg Val Met Arg Leu Val Lys Leu Leu Ser Arg Gly
50 55 60

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Trp Asn Thr Phe Asp Ser Leu Ile Val Ile Gly Ser Ile Ile Asp
1 5 10 15
Val Ala Leu Ser Glu Ala Asp Asn Ser Glu Glu Ser Asn Arg Ile Ser
20 25 30
Ile Thr Phe Phe Arg Leu Phe Arg Val Met Arg Leu Val Lys Leu Leu
35 40 45
Ser Arg Gly
50

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 66 amino acid residues
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Trp Asn Thr Phe Asp Ser Leu Ile Val Ile Gly Ser Ile Ile Asp
1 5 10 15
Val Ala Leu Ser Glu Ala Asp Pro Ser Asp Ser Glu Asn Ile Pro Leu
20 25 30
Pro Thr Ala Thr Pro Gly Asn Ser Glu Glu Ser Asn Arg Ile Ser Ile
35 40 45
Thr Phe Phe Arg Leu Phe Arg Val Met Arg Leu Val Lys Leu Leu Ser
50 55 60
Arg Gly
65

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCCGGATCCA TCGTCACCTT CCAGGAGCA

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:	
ATGGAATTCTG CCACRAAGAG GTTGATGAT	2 9
(2) INFORMATION FOR SEQ ID NO:15:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 30 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:	
GTGGGAATTC ATCAAGTCCT TCCAGGCCCT	3 0
(2) INFORMATION FOR SEQ ID NO:16:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 30 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:	
CAGGGGATCC AAGTTGTCCA TGATAACAGC	3 0
(2) INFORMATION FOR SEQ ID NO:17:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:	
CCCGAATTCA KMGTTGTGAG CATGATGAG	2 9

What is claimed is:

1. An antisense oligonucleotide that hybridizes with a region of a voltage-gated calcium channel α_1 subunit mRNA and inhibits strain-induced increase in whole cell conductance in osteoblasts, having a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

2. The antisense oligonucleotide of claim 1, which is DNA.

3. The antisense oligonucleotide of claim 1 in which at least one of the internucleotide linkages is a phosphorothioate phosphodiester.

4. The antisense oligonucleotide of claim 1 further characterized in that it does not hybridize with a CaCh1 or CaCh3 mRNA.

5. A pharmaceutical composition comprising at least one antisense oligonucleotide that hybridizes with a region of a voltage-gated calcium channel α_1 subunit mRNA and inhibits strain-induced increase in whole cell conductance in osteoblasts, having a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3, and a pharmaceutically acceptable carrier.

6. The pharmaceutical composition of claim 5, wherein the antisense oligonucleotide is DNA.

7. The pharmaceutical composition of claim 5, wherein at least one of the internucleotide linkages is a phosphorothioate phosphodiester.

8. A method of inhibiting expression of a voltage-gated calcium channel α_1 subunit gene in a cell, the method comprising the steps of:

(a) obtaining an antisense oligonucleotide that hybridizes with a region of a voltage-gated calcium channel α_1 subunit mRNA and inhibits expression of said α_1 subunit, having a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3,

(b) combining said antisense oligonucleotide with a pharmaceutically acceptable carrier to create a pharmaceutically active mixture, and

(c) contacting said voltage-gated calcium channel α_1 subunit mRNA within said cell in vitro with said pharmaceutically active mixture, thereby inhibiting expression of said voltage-gated calcium channel α_1 subunit gene within said cell.

9. The method of claim 8, wherein said cell is an osteoblast or an endothelial cell.

10. A method of inhibiting expression of a voltage-gated calcium channel α_1 subunit in a cell, comprising contacting said cell in vitro with an oligonucleotide having the sequence shown in SEQ ID NO:2 or SEQ ID NO:3.

11. The method of claim 10, wherein said cell is an osteoblast or an endothelial cell.